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In vitro biodegradability and mechanical properties of bioabsorbable bacterial cellulose incorporating cellulases

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ABSTRACT

Bacterially produced cellulose is being actively studied as a novel scaffold material for wound care and tissue engineering applications. Bioabsorbability of the scaffold material is desired to enable improved restoration of targeted tissue. Recently, a bioabsorbable bacterial cellulose (BBC) incorporating cellulase enzymes has been demonstrated. It was revealed that some cellulases may lose up to 90% of their activity if present in a suboptimal pH environment. Therefore, a key challenge in the practical implementation of this approach rests in compensating for the variation in the wound or tissue pH, which may significantly reduce the activity of some enzymes. In this work, buffer ingredients were incorporated into the bacterial cellulose in order to create a more optimal pH microenvironment for the preferred acid cellulases, which are significantly less active at the biological pH 7.4. The results demonstrated that incorporation of buffer ingredients helped to retain the activity of the cellulases. The glucose released from degraded materials was also increased from 30% without incorporation of buffer ingredients to 97% in the presence of incorporated buffer ingredients at the suboptimal pH environment of 7.4. The use of simulated body fluid and simulated tissue padding, both mimicking the real wound environment, also demonstrated some improvements in terms of material degradation. Measurements of mechanical properties of materials revealed that BBC materials have tensile strength and extensibility similar to human skin, especially when hydrated with saline water prior to use.

1. Introduction

The development of bioabsorbable tissue scaffold materials is a topic of intense research. Such a material is sought for many medical applications, ranging from wound healing to skin, bone, cartilage, nerve and other tissue regeneration. Synthetic polymers have been extensively examined and implemented as tissue scaffold materials, but many exhibit undesirable characteristics including non-bioabsorbability and low biocompatibility. Although naturally derived polymers are being implemented as alternative materials due to their high biocompatibility, some are not bioabsorbable or elicit undesirable side effects including infection and immune response [1]. Some improved materials recently examined, such as siliceous fiber [2,3] and naturally derived materials such as small intestine submucosa membranes [4,5], offer improved performance, but still exhibit several issues including possible side effects of impurities or vestigial degradation products in the body, non-controlled degradation periods, and unwanted heterogeneous compounds that may result in a rejection response.

Bacterial cellulose, a naturally derived material, has become a promising material for wound care and tissue engineering applications and has been made into commercial products (Biofill, Gengiflex and Xcell) since the 1980s [6,7]. It exhibits many desirable properties, including biocompatibility and excellent fluid exchange capabilities [8,9]. Diverse physical morphologies of bacterial cellulose synthesized in the presence of different molded substrates enable it to be directly used as the scaffold or temporary organ/tissue substitutes [7]. A recent discovery of the unique morphology of sphere-like bacterial cellulose particles may expand the application scope of bacterial cellulose for biomedical and other applications [10]. Bacterial cellulose, due to its composition consisting entirely of glucose monomers, may be an ideal bioabsorbable material. However, it cannot achieve bioabsorption in the human body because there is no chemical or enzymatic process that can cleave the β-1,4 glycosidic linkage and convert the crystalline cellulose fibrils into glucose. Such an enzymatic process exists in nature and it is utilized by bacteria, fungi and insects [11,12]. Accordingly, the realization of in-body degradation of cellulose can hardly be achieved unless through the use of enzymes, as shown previously [13]. Cellulose-degrading enzymes, referred to as cellulases, may give rise to complete degradation of cellulose into glucose [14]. Cellulases are also industrially categorized into four groups based on their active pH range: acid cellulases (pH 4.5–5.0), hybrid cellulases (pH 4.5–7.0), neutral cellulases (pH 6.0–8.0) and alkaline cellulases (pH 7.2–8.5) [15,16]. It is well

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known that wound healing displays a range of pH 5.5–7.8 during the first several days and then remains relatively stable within a pH range of 7.4–7.6 [17,18]. One approach to maintaining the activity of cellulases in the context of wound pH is to select cellulases from the hybrid or neutral/alkaline groups. However, previous studies [13] showed that bacterial cellulose containing commercial neutral/alkaline cellulases failed to show good degradability at a pH value above 6.0, suggesting that common neutral and alkaline cellulases may not be able to effectively hydrolyze bacterial cellulose or are impure. Bacterial cellulose containing acid or hybrid cellulases could achieve degradation at a pH value above 7.0, although the degradability was much lower than it was at their optimal pH value below 6.0. Previous studies also showed that cellulases preserved under a suboptimal pH value for a long period of time (4 weeks) would lose almost 90% of their original activity [13]. As a result, an alternative approach may be employed to create a suitable pH microenvironment for the cellulases, allowing them to maintain their optimal activities.

In this work, a double lyophilization (freeze-drying) process was used to make bioabsorbable bacterial cellulose (BBC) materials. Buffer ingredients were introduced into the bacterial cellulose along with cellulases to provide an optimal pH microenvironment when materials were hydrated before use. A simulated body fluid (SBF) buffer was used to create a physiologically relevant in vitro environment for degradation studies [19]. In addition, the mechanical properties of materials studied were measured. The collagen present in the dermis generally contributes to the mechanical properties of skin [20]. Common wound-care materials generally exhibit an average tensile strength of 0.42 MPa, comparable to human skin (0.02–18 MPa) [21,22]. Therefore, a desirable wound-care material should have mechanical properties similar to collagen, including low extensibility (rupture at strains on the order of 5–6%) and high modulus (approximately 0.1–1 GPa in the linear region) [23]. In this work, BBC containing pH buffering ingredients and cellulases was expected to exhibit mechanical properties similar to those of human skin.

2. Materials and methods

2.1. Materials

Bacterial cellulose pellicles were harvested from the static cultivation of *Gluconacetobacter xylinum* (ATCC 700178).

Cellulases were obtained from Sigma and were directly used without further purification. Enzyme sources and characteristics are shown in Table 1.

Polyhexamethylene biguanide (PHMB) provided by Bayer Innovation (Germany) was added to bacterial cellulose materials as a biosanitizer [24].

2.2. Production and purification of bacterial celluloses

G. xylinum (ATCC 700178) was used to synthesize bacterial cellulose pellicles in a pH 5.0 nutrient medium buffered by 3 M HCl. One liter of nutrient medium consisted of 20.0 g glucose, 5.0 g yeast extract, 5.0 g bacterial peptone, 2.7 g sodium phosphate dibasic, 1.2 g citric acid, 1.0 g magnesium sulfate, 2.0 g ammonium sulfate and 1 ml supernatant of corn steep liquor. After a 15 min autoclave, nutrient medium with 1% (v/v) bacterial inoculum was dumped into a sterile rectangular pan and then placed into a sterile incubator. A 7–8 day static cultivation at 30 °C was executed until bacterial cellulose pellicles had grown to 3–5 mm thick [25]. Longer culture times may be needed for thicker pellicles, up to 10 cm [26], as required. In addition, the yield of cellulose pellicles can be affected by many factors, such as oxygen, temperature and medium ingredients [27]. Purification of cellulose pellicles harvested from medium began with a continuous rinse with deionized (DI) water until small cellulose fragments and medium ingredients were thoroughly removed from the intact pellicle. Next, the clean pellicle was treated by 0.1 M sodium hydroxide at 80 °C for 1 h to lyse the bacterial cells. The alkaline treatment was repeated at least three times to ensure all the cells had been broken. Subsequently, cellulose pellicles were washed again by DI water until sodium hydroxide and cell debris were removed. Finally, the purified cellulose pellicles were stored at 4 °C in DI water.

Table 1
Characteristics of commercial cellulosic degrading enzymes.

Enzyme	Activity and activity conditions	Sources
A (powder)	Cellulase from *Trichoderma viride*, pH 5.0, 37 °C, >5000 U[a]/g solid	Sigma C0615
B (powder)	Cellulase from *Trichoderma reesei*, pH 5.0, 37 °C, >1000 U/g solid	Sigma C8546
C (powder)	Cellulase from *Trichoderma viride*, pH 5.0, 37 °C, >3000 U/g solid	Sigma C1794

[a] U in this table is defined as the activity of the enzyme that can liberate 1 μmol of glucose in 1 h at the indicated pH value and temperature in a 2 h incubation time.

2.3. Preparation of buffers and SBF

Citric acid-sodium citrate (CA-SC) and citric acid-sodium phosphate dibasic (CA-SPD) buffers were prepared at four different pH values at 37 °C: pH 3.0, 3.5, 4.0, 4.5. CA-SC buffer consisted of 0.1 M citric acid and 0.1 M sodium citrate, and CA-SPD buffer consisted of 0.1 M citric acid and 0.2 M sodium phosphate dibasic.

SBF [28–30] solution, a physiologically relevant environmental buffer, and phosphate-buffered saline (PBS) [31] solution, used as a control, were prepared at pH 7.4 and at 37 °C. PBS solution consisted of KCl, KH_2PO_4, NaCl and Na_2HPO_4, and was buffered to pH 7.4 with 3 M HCl. PBS solution has an approximate biological osmotic pressure and a biological pH, and its ion concentrations are shown in Table 2. SBF solution was prepared by dissolving NaCl, $NaHCO_3$, KCl, $K_2HPO_4{\cdot}3H_2O$, $MgCl_2{\cdot}6H_2O$ and $CaCl_2$ into DI water and buffered to pH 7.4 at 37 °C with 50 mM trishydroxymethyl aminomethane and 45 mM HCl. It is believed that SBF has ion concentrations similar to those of human blood plasma (Table 2).

2.4. Fabrication of BBC wound materials

The purified cellulose pellicles from storage were frozen at −20 °C and then immediately put in a freeze-dryer (Labconco FreeZone 2.5 Freeze Dry Systems) and lyophilized for at least 24 h. Subsequently, the freeze-dried pellicle was cut into approximately 2.5 × 2.5 cm rectangular pieces. Five milligrams of cellulases mixed with 0.2 mg of PHMB were dissolved individually in 0.6 ml of buffers with different pH values. Enzyme solutions were then allocated via pipette to the small pieces after the steam sterilization. These small pieces were enclosed in a sealed device for approximately 15 min to allow the solution to evenly diffuse throughout the pieces. Lyophilization was used again to obtain the final BBC products.

2.5. Investigation of buffering capacity of CA-SA and CA-SPD

Cellulosic pieces were prepared by following the procedure of BBC products as described above excluding the presence of cellulases. One milliliter of buffer (CA-SA or CA-SPD) with 0.2 mg PHMB at pH 3.0, 3.5, 4.0 or pH 4.5 was introduced to 2.5 × 2.5 cm rectangular pieces and then lyophilized after a 15 min diffusion time to ensure a homogeneous distribution of the solution within the material. Dehydrated cellulosic pieces were placed in 10 ml of

Table 2
Ion concentrations of phosphate-buffered saline (PBS), simulated body fluid (SBF) and human blood plasma [28].

	Ion concentrations (mM)							
	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	HCO_3^-	$H_2PO_4^-$	HPO_4^{2-}
PBS	153.1	4.2	–	–	139.6	–	1.5	8.1
SBF	142.0	5.0	1.5	2.5	148.8	4.2	–	1.0
Human blood plasma	142.0	5.0	1.5	2.5	103.0	27.0	–	1.0

PBS or SBF solutions using two experimental configurations, respectively, as shown in Fig. 1. The first method positioned the sample such that it was in contact with, but not completely submerged in 10 ml of environmental solution. This was accomplished through the use of a porous aluminum screen (24-mesh) which suspended the sample on the interface between the environmental solution and the air. This experimental set-up was developed to better simulate the actual use of the material in a wound-care application. In the second method, the sample was completely submerged in 10 ml of the environmental solution. The pH values of the environmental solution and the surface of the samples were measured every 24 h.

2.6. Investigation of simulated in vitro degradation of BBC samples

Based on the measured buffering capacity of CA-SC and CA-SPD, the optimal pH value for the selected buffers was determined to create an appropriate microenvironment where cellulases would work optimally. After creating the final BBC samples, simulated in vitro degradation studies were conducted in two different environmental solutions, PBS (pH 7.4) and SBF (pH 7.4). This set of biodegradation experiments was executed in terms of the two experimental configurations as described above. The experimental results were evaluated by visual observation every day until the seventh day according to the degradation degrees described as none (■; no degradation), slight (■; very little but visible degradation), moderate (■; at least ~50% of sample was no longer visible), extensive (■; ~90% of sample disappeared and it had been decomposed from a single large whole to small patches), nearly complete (▬; ~100% of sample vanished but many smaller particles appeared and the solution looked cloudy) and complete (□; no visible particles presented and the solution looked nearly transparent).

2.7. Sugar analysis

Glucose and cello-oligosaccharides possibly present in cellulose hydrolysate in the environmental solution were analyzed [32] by high-performance liquid chromatography (HPLC) (Shimadzu, Japan) using a Shodex NHP52 column. DI water was set as mobile phase A and pure acetonitrile (ACN) was set as mobile phase B. The concentration of mobile phase B was initially set at 75% and gradually varied to 35% within 8 min, and then was maintained at 35% for 0.5 min. The total running time was 10 min for each sample. Cello-oligosaccharide standard was purchased from Sigma (C8071) and consists of cellobiose, cellotriose, cellotetraose and cellopentaose.

Glucose released from the bacterial cellulose in the environmental solution on days 1, 4 and 7 was measured using a YSI 2700 Biochemistry Analyzer (YSI Inc., USA) and the following equations were used to determine the percentage of actual glucose yield to maximally ideal glucose yield.

$$\text{Maximally ideal glucose released} = \frac{\text{weight of dehydrated material without enzymes} \times 180}{162} \quad (1)$$

$$\text{Ratio} = \frac{\text{actual glucose yield}}{\text{maximally ideal glucose yield}} \quad (2)$$

where 180 is the molecular weight of glucose, and 162 is the molecular weight of anhydroglucose as one glycosidic unit of cellulose.

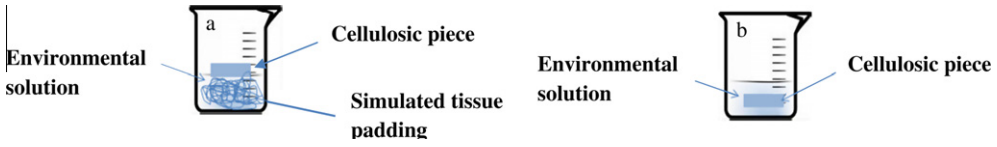


Fig. 1. Two experimental configurations set for studying the degradation of BBC samples where the sample is (a) positioned at the air–solution interface and (b) submerged in the solution.

Table 3
Buffering capacity of CA-SC in PBS and SBF.

CA-SC		pH 3.0				pH 3.5				pH 4.0				pH 4.5			
		Method 1[a]		Method 2[b]		Method 1		Method 2		Method 1		Method 2		Method 1		Method 2	
		S[c]	E[d]	S	E	S	E	S	E	S	E	S	E	S	E	S	E
10 ml PBS	0 h	3.5	6.0	3.5	4.5	4.0	6.5	4.0	5.0	4.5	6.5	5.0	5.5	5.0	7.0	5.0	6.0
	24 h	3.5–4.0	4.0	3.5	4.0	4.0–4.5	4.0–4.5	4.5	4.5	4.5–5.0	4.5–5.0	5.0	5.0	5.0–5.5	5.0–5.5	5.0–5.5	5.0–5.5
	48 h	3.5–4.0	3.5–4.0	4.0	4.0	4.0–4.5	4.0–4.5	4.5	4.5	4.5–5.0	4.5–5.0	5.0	5.0	5.0–5.5	5.0–5.5	5.0–5.5	5.0–5.5
10 ml SBF	0 h	3.5–4.0	5.0	3.5–4.0	4.5	4.0	5.0	4.0	5.0	4.5	5.0	4.5	5.0	5.0	6.0	5.0	5.5
	24 h	4.0	4.0	4.0	4.0	4.0–4.5	4.0–4.5	4.0–4.5	4.0–4.5	4.5–5.0	4.5–5.0	4.5–5.0	4.5–5.0	5.0	5.0	5.0	5.0
	48 h	4.0	4.0	4.0	4.0	4.0–4.5	4.0–4.5	4.0–4.5	4.0–4.5	4.5–5.0	4.5–5.0	4.5–5.0	4.5–5.0	5.0	5.0	5.0	5.0

[a] Method 1: sample pieces positioned at the air–solution interface by a simulated tissue padding.
[b] Method 2: sample pieces submerged in the solution.
[c] S: pH value of piece's surface.
[d] E: pH value of environmental solution.

Table 4
Buffering capacity of CA-SPD in PBS and SBF.

CA-SPD		pH 3.0				pH 3.5				pH 4.0				pH 4.5			
		Method 1[a]		Method 2[b]		Method 1		Method 2		Method 1		Method 2		Method 1		Method 2	
		S[c]	E[d]	S	E	S	E	S	E	S	E	S	E	S	E	S	E
10 ml PBS	0 h	3.5–4.0	6.0	4.0–4.5	5.5	4.0	6.0–6.5	4.5–5.0	6.0	4.5–5.0	6.5	4.5–5.0	6.5	5.0–5.5	6.5–7.0	5.0–5.5	6.5–7.0
	24 h	4.5	4.0–4.5	4.5	4.0–4.5	5.0–5.5	5.0–5.5	5.0	5.0	5.5	5.5	5.5	5.5	6.0	6.0	6.0	6.0
	48 h	4.0–4.5	4.0–4.5	4.0–4.5	4.0–4.5	5.5	5.5	5.0	5.0	5.5	5.5	5.5	5.5	6.0	6.0	6.0	6.0
10 ml SBF	0 h	3.5	5.0	3.5	5.0	4.0–4.5	6.0	4.0–4.5	5.0	4.5–5.0	6.0	4.5–5.0	5.5–6.0	5.0	6.0	5.0	6.0
	24 h	4.0	4.0	4.0	4.0	4.5–5.0	4.5–5.0	4.5–5.0	4.5	5.0	5.0	5.0	5.0	5.0–5.5	5.0–5.5	5.0–5.5	5.0–5.5
	48 h	4.0	4.0	4.0	4.0	4.5–5.0	4.5–5.0	4.5–5.0	4.5–5.0	5.0	5.0	5.0	5.0	5.0–5.5	5.0–5.5	5.0–5.5	5.0–5.5

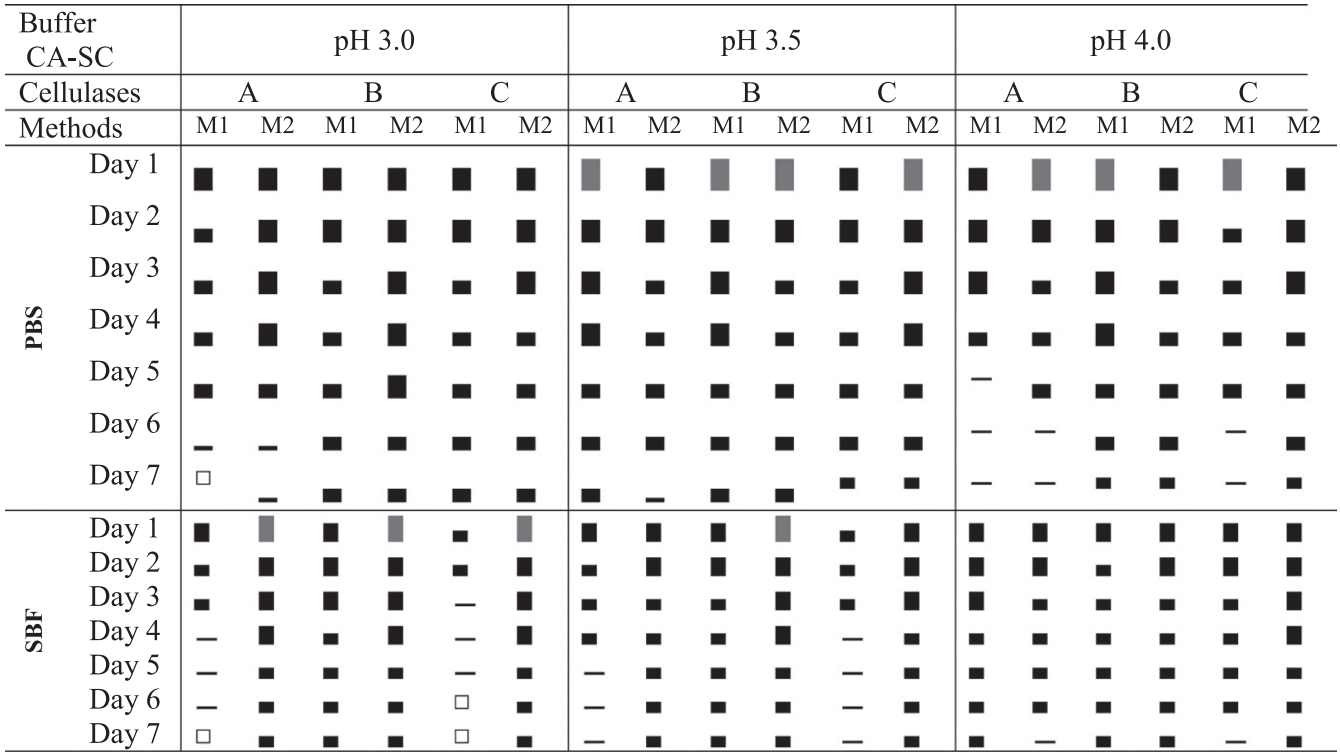
[a] Method 1: sample pieces positioned at the air–solution interface by a simulated tissue padding.
[b] Method 2: sample pieces submerged in the solution.
[c] S: pH value of piece's surface.
[d] E: pH value of environmental solution.

2.8. Mechanical properties of cellulosic pieces

Four different dehydrated 2.5 cm × 2.5 cm samples were prepared using the procedure described above. Sample 1 was a control sample hydrated with only DI water; sample 2 contained both buffer ingredients and PHMB but no enzymes; sample 3 contained enzymes and PHMB but no buffer ingredients; and sample 4 contained the complete combination of buffer ingredients, enzymes and PHMB. Mechanical properties for dehydrated samples and rehydrated samples were measured by dynamic mechanical analysis (DMA) using a TA Instruments Q800 analyzer. The rehydrated samples were prepared by soaking dehydrated samples in saline water (0.9% NaCl) for 1 min prior to DMA testing. A simple and rapid experiment showed that the rehydrated samples in the air-tight DMA furnace were only minimally dehydrated at 37 °C during the measurement. Several dehydrated samples were rehydrated and sealed with parafilm and placed in a 37 °C environment for 1 h; the differences in weight of the samples before and after placing them in this environment were measured. The results showed an approximately 3.5% weight loss, suggesting that rehydrated samples were able to remain hydrated during the measurement.

The mechanical properties measured included stress (MPa) and strain (%) at break, as well as the Young's modulus, which was calculated from the data of stress and strain in the linear region (GPa). Here, stress is F/A, where F is the loading force and A is the area of the cross-section of materials, calculated from the width multiplied by the thickness. Strain is simply $\Delta L/L$, where

Table 5
Description of degrees of degradation of BBC pieces in PBS and SBF.



Key: none (■; no degradation), slight (▓; very little but visible degradation), moderate (▓; at least ~50% of sample was no longer visible), extensive (■; ~90% of sample disappeared and it had decomposed from a single large whole to small patches), nearly complete (▬; ~100% of sample vanished but many smaller particles appeared and the solution looked cloudy) and complete (□; no visible particles presented and the solution looked nearly transparent).
[a] M1: Method 1, sample pieces positioned at the air–solution interface by a simulated tissue padding.
[b] M2: Method 2, sample pieces submerged in the solution.

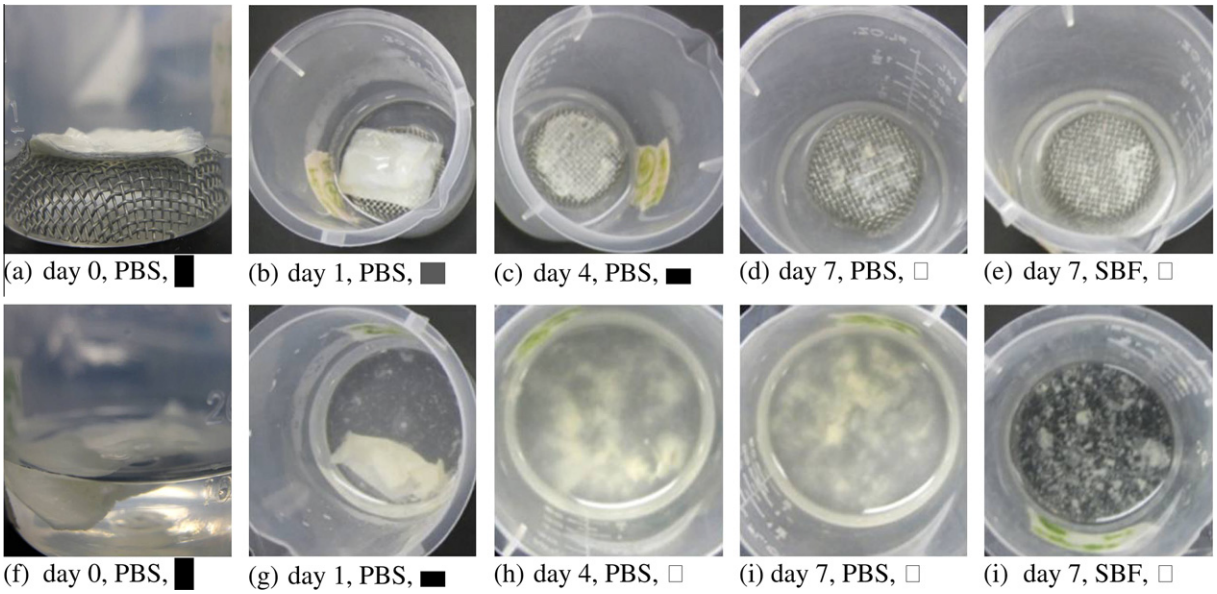


Fig. 2. Macroscopic pictures of degradation of BBC pieces incorporating cellulase B and buffer ingredients with pH 4.0 in PBS and SBF. (a–e) Degradation in the presence of simulated tissue padding; (f–i) degradation without simulated tissue padding. Degradation levels: none (■), slight (■), moderate (■), extensive (■), nearly complete (▬) and complete (□).

L is the initial length and ΔL is the difference between the lengths at break and the initial length. All the samples were cut to a size of 20 mm × 3–5 mm (length × width) and the thickness was measured prior to loading the samples. Loading forces were changed at a rate of 0.2 N min^{-1} and the temperature was kept at 37 °C.

3. Results and discussions

A challenge in understanding the degradation behavior of the BBC materials described in this work lies in quantifying the amount of tissue fluid to which samples will be exposed in practical applications. It is difficult to quantify the volume of tissue fluid

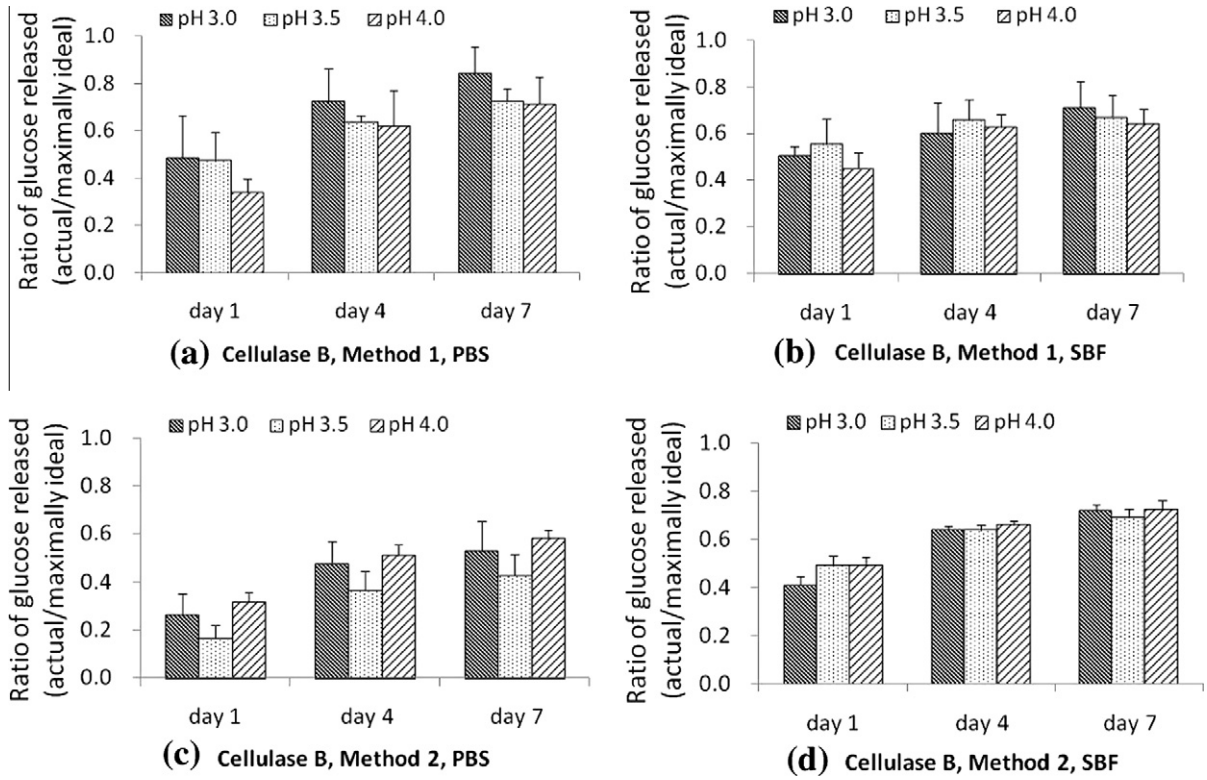


Fig. 3. Ratios of glucose released from degradation of BBC materials containing cellulase A: (a) in PBS using method 1; (b) in SBF using method 2; (c) in PBS using method 1; (d) in SBF using method 2. Method 1: BBC pieces located on the interface between the air and the environmental solution by a simulated tissue padding as described in Fig. 1. Method 2: BBC pieces totally submerged in the environmental solution as described in Fig. 1.

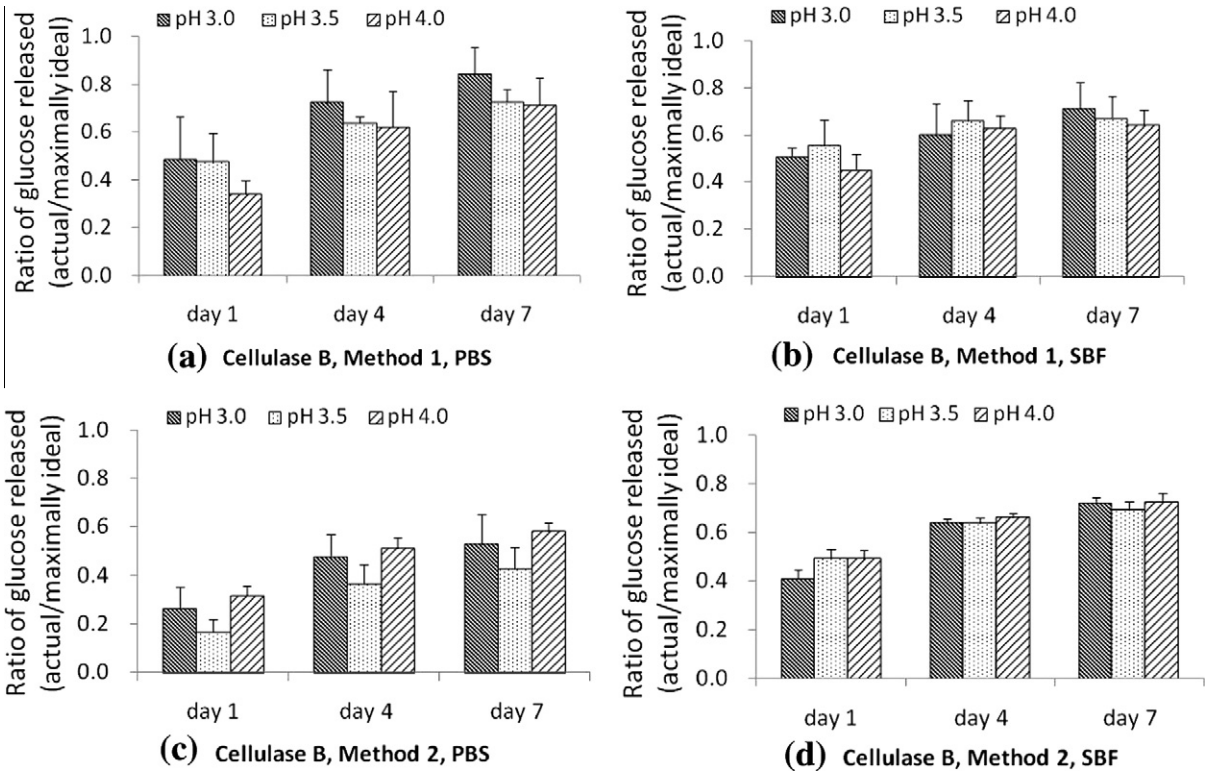


Fig. 4. Ratios of glucose released from degradation of BBC materials containing cellulase B: (a) in PBS using method 1; (b) in SBF using method 2; (c) in PBS using method 1; (d) in SBF using method 2. Method 1: BBC pieces located on the interface between the air and the environmental solution by a simulated tissue padding as described in Fig. 1. Method 2: BBC pieces totally submerged in the environmental solution as described in Fig. 1.

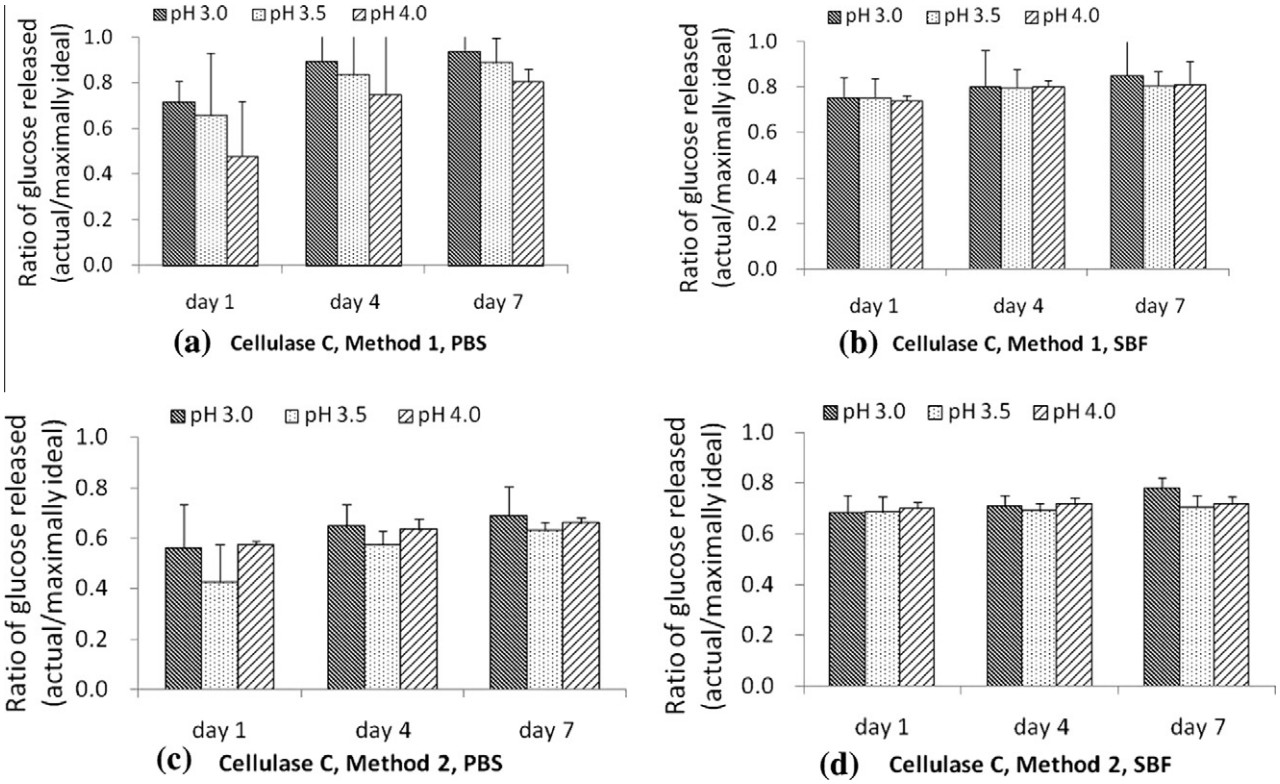


Fig. 5. Ratios of glucose released from degradation of BBC materials containing cellulase C: (a) in PBS using method 1; (b) in SBF using method 2; (c) in PBS using method 1; (d) in SBF using method 2. Method 1: BBC pieces located on the interface between the air and the environmental solution by a simulated tissue padding as described in Fig. 1. Method 2: BBC pieces totally submerged in the environmental solution as described in Fig. 1.

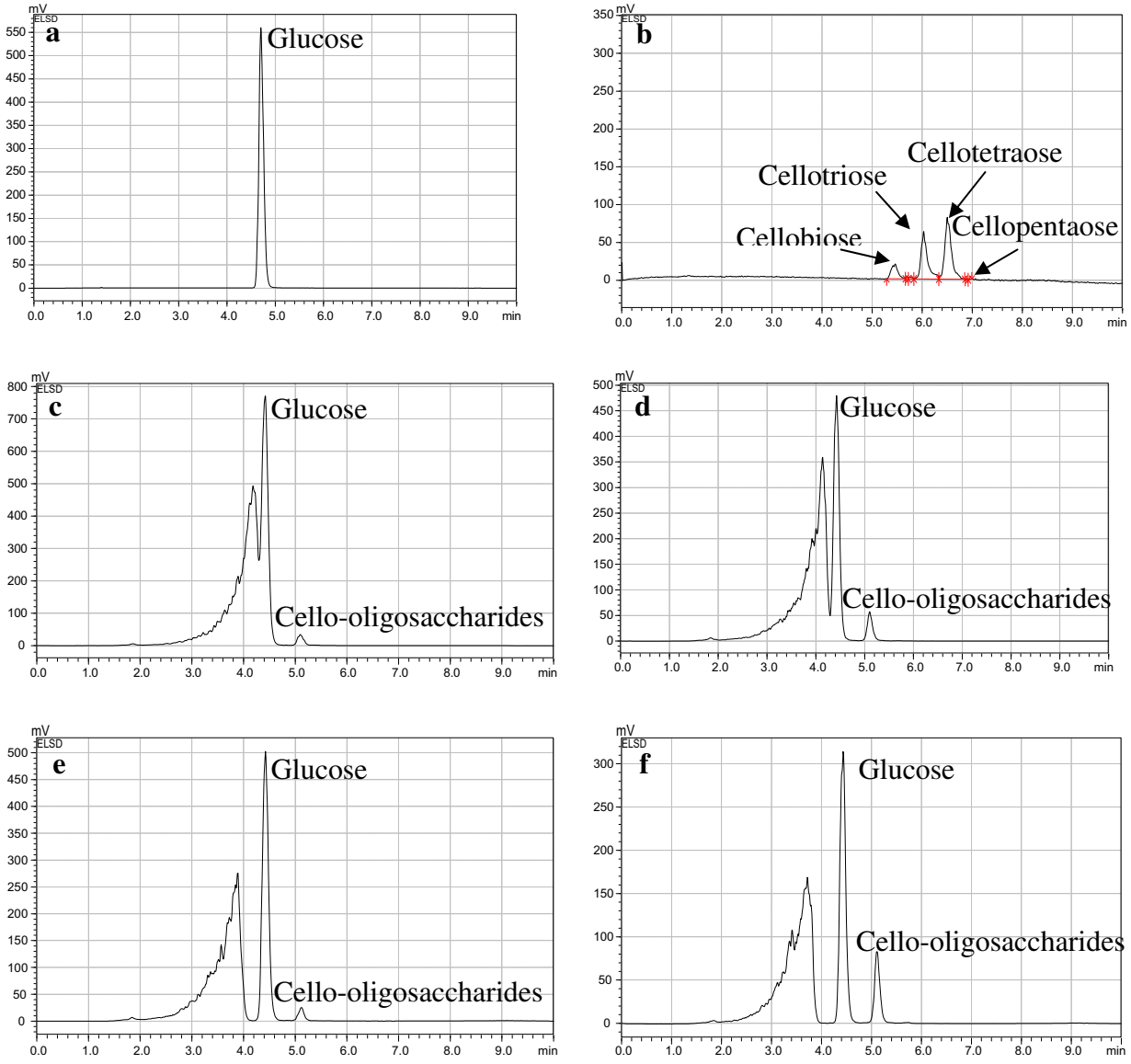


Fig. 6. HPLC chromatographs of degradation hydrolysates of BBC pieces containing cellulase B in the presence of simulated tissue padding on day 7: (a) standard glucose; (b) standard cello-oligosaccharides including cellobiose, cellotriose, cellotetraose and cellopentaose; (c) when buffered to a pH of 3.0 in PBS; (d) when buffered to a pH of 4.0 in PBS; (e) when buffered to a pH of 3.0 in SBF; (f) when buffered to a pH of 4.0 in SBF.

in real wound environments as this varies over time. Estimation can be made based on the volume of capillaries under the epidermis as well as the blood flow rate in the capillaries. The volume of capillaries is estimated to be only 3.5% of tissues based on experiments conducted on rats, and a blood flow rate of around 0.14–0.94 mm s^{-1} in tissue capillaries has been demonstrated [33–35]. When injuries occur, an interface between medical materials and fluid flowing out from damaged tissue begins to form. Interactions between materials and wounds at this interface are extremely important where medical materials offer pressure to block bleeding and then form a temporarily stable enclosure. At this time, the flow rate of blood from the damaged tissue is forced to decrease and the tissue fluid remains relatively stable. Although we are unable to find in the literature any quantitative measure of the amount of fluid produced in wounds as they heal, we hypothesize that a wound of roughly the size of our sample would not be exposed to more than 5–10 ml of tissue fluid. Thus in these experiments we selected 10 ml of solution to simulate the real wound environment.

It is known that wound pH values vary over time from 5.5 to 7.8, but after prolonged periods will settle at pH 7.3–7.6, especially for chronic wounds [36]. Studies have demonstrated that acid cellulases exposed to suboptimal pH buffers (6.5–7.0) would exhibit a significant decrease in activity [13]. In addition, wound-care materials must provide adequate mechanical support until tissue cells begin to proliferate, thus requiring a gradual or delayed degradation period. Control over the degradation rate can be achieved by stabilizing the wound pH to a desired value and selecting an appropriate enzyme concentration (assuming the enzyme activity remains invariant, as shown for a specific cellulase identified in Ref. [13]) given the activity of the enzyme at that pH value.

In this work, we stabilized the pH of the simulated wound microenvironment by using two buffers, CA-SC and CA-SPD, as shown in Tables 3 and 4, respectively. Buffer CA-SC showed a higher buffering capacity in two environmental solutions, PBS and SBF: it could maintain a pH surrounding the cellulosic sample pieces in the range 4.5–5.0 when the samples were submerged in solutions at pH values of 3.5, 4.0 and 4.5 for 48 h. Although buffer CA-SPD

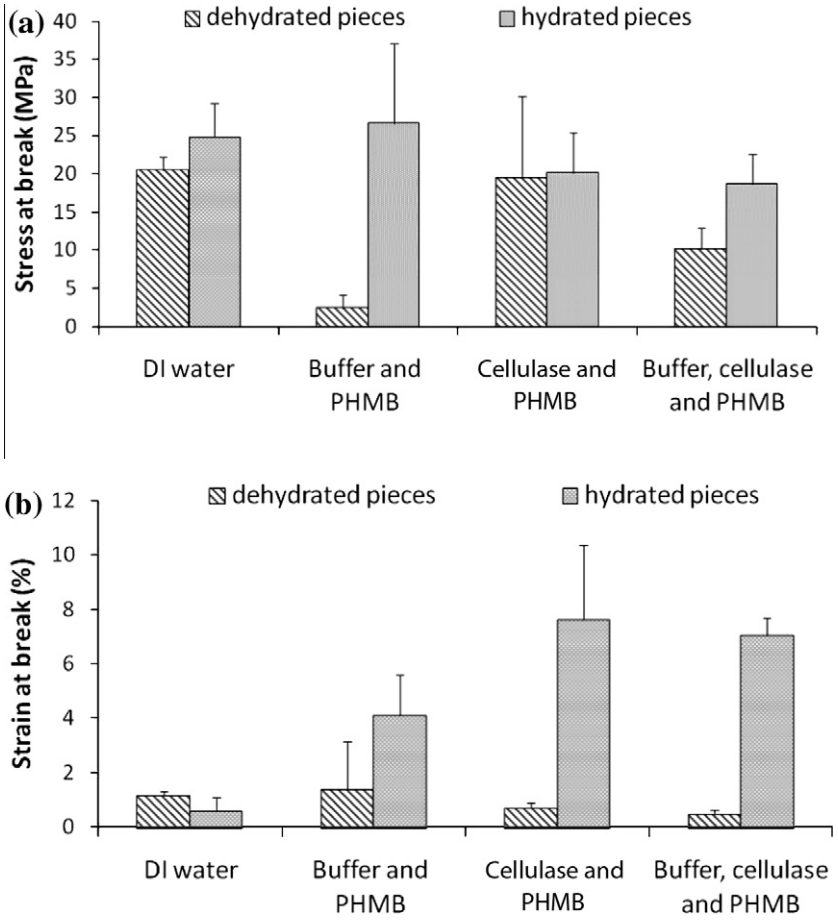


Fig. 7. (a) Stress and (b) strain comparisons of dehydrated sample pieces and hydrated pieces in the presence of DI water; buffers and PHMB; DI water, enzyme and PHMB; buffers, enzyme and PHMB.

also showed good buffering capacity in the pH range 4.5–5.5, buffer CA-SC might be the first choice because the phosphate element in CA-SPD may sequester divalent cations, e.g. calcium and magnesium, which may partly inhibit enzymatic reactions [37]. The pH values on/around sample pieces after 48 h did not show significant differences for the two experimental configurations shown in Fig. 1.

Based on the results of the buffering capacity experiments, buffer CA-SC prepared at initial pHs of 3.0, 3.5, 4.0 was selected to be incorporated to the cellulosic sample pieces with cellulases and PHMB. This buffer exhibited the most stable pH with variations in pH of the PBS and SBF buffers. The degradations were performed in 10 ml PBS and 10 ml SBF, respectively, using two different experimental configurations as shown in Fig. 1. The evaluation of different degradations during a period of 7 days is described in Table 5. In general, samples incorporating cellulase B in the CA-SC buffer exhibited the slowest rate of degradation. For a CA-SC buffer pH of 3.0, 50% of the samples incorporating cellulase B in PBS solution were visible after 5 days. The use of aluminum-made simulated tissue padding appeared to affect the degradation. Denser fragments of cellulose were visible on the surface of the simulated tissue padding for days 4–7 in the case of the PBS buffer (Fig. 2d–e), suggesting that the tissue padding may hold the cellulose materials together during the degradation process. These denser fragments may provide additional mechanical integrity for cell growth. Sample pieces incorporating cellulase B and buffer ingredients with pH 3.0 exhibited the best mechanical integrity over 5 days. It has been demonstrated [13] that cellulase B has a relatively long lifetime as well as a relatively low activity, which implies that cellulase B might slowly degrade cellulosic materials over a longer period of time. However, most sample pieces observed in degradations lost more than 50% of their integrity after only 1 day, possibly due to the high cellulase loading used in this study. In terms of cell growth, the BBC samples need to retain at least 80% of their integrity until day 2 as human skin fibroblast requires a substrate to attach to and quickly proliferate on after injuries occur [38]. We believe that this rapid degradation rate of sample pieces in this study could be controlled by using lower loadings of cellulase B once the relatively optimal pH microenvironment for cellulases B was created, as cellulase B exhibited a low and stable activity.

Figs. 3–5 show measured glucose released from the degradation of BBC pieces in the environmental solutions. Although some error bars present in Figs. 3–5 were significant, differences between the set pH values 3.0, 3.5 and 4.0 were not generally observed for cellulases B and C. For samples incorporating cellulase A tested using method 1, a decrease in glucose released was observed between pH 3.0 and 4.0 on day 1 in PBS as compared to SBF, however this difference amongst set pH values appeared to vanish on the last tested day (day 7). For samples incorporating cellulase A tested using method 2, a decrease in glucose release was also observed for all pH values tested on day 1 in PBS as compared to SBF. However, on the last tested day (day 7), slightly more glucose was released in samples with set pH values of 3.0 and 3.5 in the PBS buffer. In general, however, the data shown in Figs. 3–5 indicates that cellulase B in samples prepared using method 2 provided the most gradual rate of glucose release and lower total glucose released. In addition, it was shown that cellulase B was able to maintain its activity for a minimum of 30 days [13], indicating that

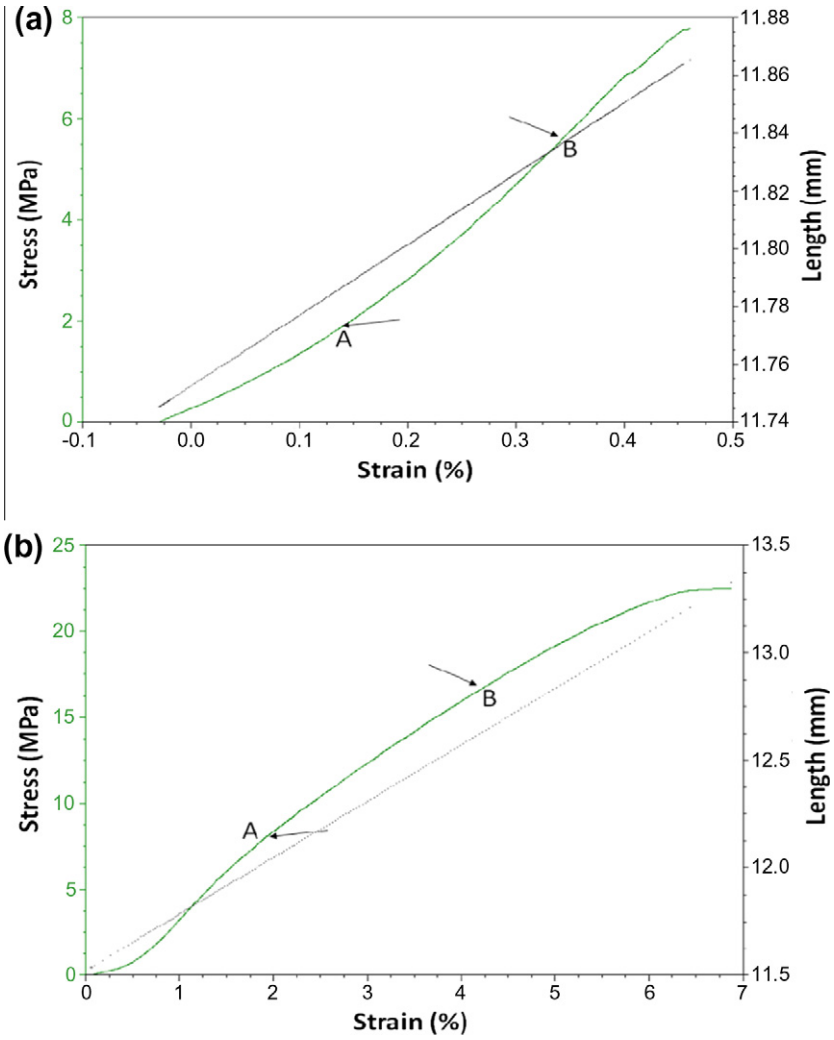


Fig. 8. Young's modulus shown in the linear region between point A and point B on the curve for (a) a dehydrated piece and (b) a hydrated piece.

degradation of the samples would continue beyond day 7. This suggests that cellulase B might be used for applications requiring longer-term degradation if the appropriate enzyme loading was used. When selecting cellulases, both long-term stability and activity are key points, i.e. lower activity provides more flexibility in regulating degradation via changes in concentration.

We have mentioned that although the wound pH varies over time from 5.5 to 7.8, low pH only occurs in the first couple of days, and afterwards the pH will stabilize above 7.0. Previous studies have shown [13] that BBC materials with cellulases A, B and C, which did not contain buffer ingredients, released a maximum of 30% of the total possible glucose at an environmental pH 7.4. However, when incorporating buffers into the sample pieces in this work, these materials released at least 43% of the total possible glucose at an environmental pH 7.4 (BBC pieces with cellulase B and pH 3.5 buffer ingredients, Fig. 4c) and as much as 97% (BBC pieces with cellulase A and pH 3.0 buffer ingredients, Fig. 3a). These results suggest that incorporating buffer ingredients stabilized the solution pH of 7.4 to a pH appropriate for good cellulase activity.

HPLC sugar analysis revealed that a small amount of cello-oligosaccharides was present in hydrolysates of BBC pieces containing cellulase B as shown in Fig. 6c–f. In the presence of simulated tissue padding, loaded buffer ingredients with lower pH values appeared to release more glucose and less cello-oligosaccharide remained, consistent with improved enzyme activity at lower pH values. In general, method 1, involving the use of simulated tissue padding, provided a higher glucose release compared to method 2, where the sample was completely submerged. We hypothesize that in this case, limited out-diffusion of the enzyme on the top surface of the sample provided a higher concentration of enzyme within the sample. However, the largest difference in glucose released on day 7 between samples examined using methods 1 and 2 ranged from 43% (BBC pieces with cellulase B and pH 3.5 buffer ingredients using method 2) to 97% (BBC pieces with cellulase A and pH 3.5 buffer ingredients using method 1). This suggests that enzyme diffusion from the sample has a limited impact on the degradation dynamics under these testing conditions.

Ideally, BBC materials should exhibit mechanical properties similar to skin or other tissues. Skin exhibits the following mechanical properties: 1.5–3.5 MPa stress at break, 5–6% strain at break and 0.1–1 GPa Young's modulus [23,39]. Data from mechanical properties testing in Fig. 7 showed that either dehydrated samples or hydrated samples exhibited strength (stress at break) and extensibility (strain at break) of the sample pieces incorporating buffer ingredients, cellulase B and PHMB comparable to these requirements (stress >3.5 MPa and strain ~6%). Sample pieces containing all needed components tested in the hydrated state exhibited high stress at break (18.65 MPa) and good extensi-

bility at break (7.04%). The presence of buffer ingredients dramatically reduced the stress at break of dehydrated samples from 19.5 to 2.4 MPa for samples without incorporated cellulase, or 10.2 MPa for the samples with incorporated cellulase. Hydration of these samples increased the stress at break to 26.6 MPa (without cellulase) and 18.6 MPa (with cellulase), respectively. Although the Young's modulus as shown in Fig. 8 before and after hydration varied, these values (1.875 GPa for dehydrated samples and 0.381 GPa for hydrated samples) were still within in the recommended range (0.1–1 GPa). These results suggest that hydration prior to the use of BBC products was very important, not only for activating the enzymes but also for providing the sample pieces with desirable mechanical properties for wound-care applications.

4. Conclusion

Bioabsorbable bacterial cellulose materials incorporating biologically compatible buffer ingredients to maintain desirable pH levels during degradation have been developed and tested. The buffer CA-SC showed optimal buffering capacity in SBF similar in composition to human blood plasma. BBC pieces containing cellulase B showed a relatively progressive degradation compared with pieces containing cellulase A or C due to its relatively low activity. All BBC pieces tested in the presence of simulated tissue padding exhibited faster degradation performance, suggesting that limited diffusion of cellulases from the top surface of the sample resulted in a higher cellulase concentration in the samples. However, the largest difference in glucose released on day 7 between samples examined with and without simulated tissue padding ranged from 43% to 97%, suggesting that enzyme diffusion from the sample has a limited impact on the degradation dynamics under these testing conditions. Although most of the BBC pieces in this study lost 80% of their integrity after 2 days, we believe that the degradation rate can be controlled by loading lower amount of cellulases to ensure the integrity of materials until cell have attached completely and begun to proliferate. High levels of glucose released from BBC pieces at pH 7.4 in this study also demonstrated that the incorporation of buffer ingredients into materials helps to form an optimal pH microenvironment for cellulases. Compared with previous studies, the released glucose was increased from approximately 30% without incorporated buffer ingredients to 97% in the presence of buffer ingredients at an environmental pH 7.4. Analysis of mechanical properties revealed that BBC samples containing all needed components have high strength (18.65 MPa), good extensibility (7.04%) and appropriate stiffness (Young's modulus 0.381 GPa) when they were hydrated using saline water (0.9% NaCl) prior to measurements. The mechanical properties of these BBC pieces meet the requirements for materials used for wound care.

Disclosure

The authors obtained financial support from Bayer to perform the work described in this manuscript. Yang Hu and Dr. Jeffrey Catchmark from the Pennsylvania State University, and Dr. Burkhard Fugmann from Bayer Innovation GmbH, Germany, are inventors on one or more patents encompassing this work. The authors and inventors report no conflicts.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figures 1, 2, 6 and 8, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.actbio.2011.03.028.

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